The diversity of aminoglycoside-modifying enzymes among ESBL - positive Proteus mirabilis clinical strains

Anna Diana Michalska, Paweł Tomasz Sacha*, Katarzyna Kaczyńska, Elżbieta Anna Tryniszewska

#Corresponding author: Paweł T. Sacha e-mail address: sachpt@umb.edu.pl

This study was supported by internal funding sources from Medical University of Bialystok (144-22822 F).

RUNNING TITLE Aminoglycoside-modifying enzymes among Proteus mirabilis

KEYWORDS aminoglycoside-modifying enzymes, Proteus mirabilis, ESBL, plasmid-mediated resistance

WORD COUNT 1 962

CONFLICT OF INTERESTS no conflicts of interest

ABSTRACT

Background: Nowadays, the spreading of multi-drug resistant bacteria poses a serious threat, which significantly reduces therapeutic options. P. mirabilis is a common etiological factor of complicated urinary tract infections. Moreover, this species can acquire resistance genes through mobile genetic elements. The most prevalent resistance mechanism among P. mirabilis strains is the production of ESBL, which may occur along with other resistance determinants. Among them, AME creates the menace of aminoglycosides effectiveness. This study was designed to investigate the co-production of AME and ESBL in clinical isolates of P. mirabilis.

Material and Methods: A total of 228 P. mirabilis strains were tested for the production of ESBL by DDST. Then, PCR with specific primer pairs was used to detect genes encoding AME among 75 ESBL-positive P. mirabilis strains.

Results: The co-production of AME along with ESBL was reported in 88% of ESBL strains. The revealed AME genes were: ant(2")-Ia (n=53), aph(3")-Ib (n=14), aac(6')-Ib (n=9), and aac(3)-Ia (n=1). Nine strains showed the presence of more than one AME gene. The most frequent combination was ant(2")-Ia+aph(3")-Ib (5 strains), followed by aac(6')-Ib+aph(3")-Ib (2 strains), and aac(6')-lb+ant(2")-la+aph(3")-Ib (2 strains). Isolates co-producing AME and ESBL showed high resistance rates to 3rd generation cephalosporins, aminoglycosides, and fluoroquinolones. Carbapenems were the only antibiotics active against all tested strains.

Conclusion: In this study we observed high prevalence of AME among P. mirabilis strains producing ESBL. The rapid dissemination of strains carrying genes for resistance to several groups of antibiotics creates the need for effective surveillance and appropriate antibiotic policy.
Proteus mirabilis, a Gram-negative rod from family Enterobacteriaceae, is one of the key pathogens associated with complicated urinary tract infections: acute pyelonephritis, bladder infections or urolithiasis [1]. This genus carries numerous virulence factors that promote colonization of the urinary tract [2]. Moreover, P. mirabilis strains can acquire various genes for antibiotic resistance via mobile genetic elements: plasmids, integrons, gene cassettes, and transposons [3]. The production of extended spectrum beta-lactamas (ESBL) is considered as one of the most important resistance mechanisms. These enzymes have the ability to hydrolyze the molecules of beta-lactam antibiotics, like 3rd generation cephalosporins (eg. cefotaxime, ceftazidime) and monobactams [4]. There are several antimicrobial options recommended for the treatment of ESBL-producing P. mirabilis – like aminoglycosides, fluoroquinolones, or carbapenems [5]. Aminoglycosides are very important group of antibiotics with activity against many Gram-negative rods [6]. Nevertheless, Gram-negative rods producing ESBLs frequently show cross-resistance with other groups of antimicrobials [7].

The most common mechanism of aminoglycoside resistance is enzymatic modification of antibiotic molecule [8]. After modification, the aminoglycoside is not able to bind to the aminoacyl site of 16S RNA within the bacterial 30S ribosomal subunits and inhibit protein synthesis [5,9]. Aminoglycoside-modifying enzymes (AME) have been assigned to three classes: aminoglycoside acetyltransferases (AAC), aminoglycoside phosphotransferases (APH), and aminoglycoside nucleotidylyltransferases (ANT) [10]. A more specific classification includes the division into subclasses according to the regiospecificities for aminoglycoside modifications and spectrum of aminoglycoside resistance [11]. To the subclasses with clinical significance among Enterobacteriaceae rods belong following groups: AAC(3)-I, AAC(6’)-I, ANT(4’)-II, ANT(2’)-I, and APH(3’)-I. The subclass AAC(3)-I contributes to the resistance to gentamicin, sisomicin, and ambracin, while AAC(6’)-I shows activity against amikacin and gentamicin. Enzymes from ANT(4’)-II subclass confer resistance to amikacin, isepamicin, and tobramycin, and AME belonging to ANT(2’)-I mediate resistance to dibekacin, gentamicin, kanamycin, sisomicin, and tobramycin. The subclass of APH(3’)-I enzymes shows activity against streptomycin [8]. The other mechanisms, which also contributes in resistance to aminoglycosides, can coexist in one bacterial cell along with AME [12]. These mechanisms include methylation of 16S rRNA [13], reduced outer membrane permeability [14], efflux pumps [15], and mutations of the ribosomal proteins or 16S rRNA [13].

In this study we screened for various subclasses of AME among ESBL-positive P. mirabilis strains obtained from patients in University Clinical Hospital of Bialystok, Poland.

MATERIAL AND METHODS

From June 2013 to June 2014, a total of 228 non-duplicated P. mirabilis strains were collected from patients hospitalized in University Clinical Hospital in Bialystok, Poland. Isolates were initially identified on the basis of colony morphology on Columbia Agar and MacConkey Agar (both from Oxoid Thermo Fisher Scientific Inc, Waltham, USA). Species identification was confirmed using the Vitek 2 Automated System with GN ID cards (bioMerieux, Marcy l’Etoile, France). All isolates were susceptibility tested by Vitek 2 System with AST cards (bioMerieux). Minimal inhibitory concentrations (MIC) of amikacin, gentamicin, and tobramycin were tested by E-tests (bioMerieux). Interpretation of susceptibility testing results was according to the newest EUCAST criteria [16]. Strains with value of MIC for cefotaxime and ceftazidime higher than 1 mg/L were selected for phenotypic ESBL confirmation [17]. Double-disc synergy test (DDST) with disks containing amoxicillin/clavulanic acid (20/10 μg), ceftazidime (30 μg), and cefepime (30 μg) (all from Oxoid) was used as phenotypic test for ESBL detection [17].

ESBL-positive strains were then selected for molecular investigation towards AME genes. Plasmid material was extracted from overnight P. mirabilis cultures by Plasmid Mini Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer’s protocol. PCR assays were performed to detect following AME genes: aac(6’)-Ib, aac(3)-Ia, ant(4’)-Ila, ant(2’)-Ia, and aph(3’)-Ib. Primers for amplification were selected from the literature (Table 1). All PCR surveys were performed in the LabCycler Gradient (SensoQuest GmbH, Goettingen, Germany) under following conditions: predenaturation step at 94°C for 5 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 52°C for 45 s and elongation at 72°C for 1 min, and a final elongation step at 72°C for 10 min. The PCR products were separated by gel electrophoresis at 5 V/cm for 110 min on a 1.5% agarose gel (Sigma-Aldrich, St. Louis, USA) containing 0.5 μg/ml of ethidium bromide (MP Biomedicals, Santa Ana, USA) in 1xTBE buffer. The analyses were performed on Sub Cell® GT apparatus (Bio-Rad, Hercules, USA). After electrophoresis amplicons were visualized under UV light and photographed using a ChemiDoc™ XR System (Bio-Rad, Hercules, USA). The sizes of the DNA fragments were calculated from their positions relative to the position of the molecular weight marker: Perfect™ 100-1000 bp DNA Ladder (EURx, Gdansk, Poland). To identify the variants of resistance genes, sequencing was performed with primers listed in Table 1 using 3500 Genetic Analyzer (Applied Biosystems, Foster City, USA). The obtained sequences were compared with sequences stored in GenBank database using the nucleotide BLAST.
algorithm.

Descriptive statistics for epidemiological purposes was performed by StataSE 12 (StataCorp LP, USA).

RESULTS

Among the 228 isolates tested by DDST, 75 (32.89%) showed the production of ESBL. These strains were selected for further investigations. The genes encoding AME were detected in 66 P. mirabilis isolates, which represents 88.0% of ESBL-positive strains. A variety of AME genes was observed: ant(2")-Ia in fifty-three, aph(3")-Ib in fourteen, aac(6')-Ib in nine strains, and aac(3)-Ia in one strain. In nine strains the presence of more than one AME gene was revealed. Coexistence of ant(2")-Ia and aph(3")-Ib was detected in five strains, while the presence of aac(6')-Ib and aph(3")-Ib was revealed in two strains, and aac(6')-Ib with ant(2")-Ia and aph(3")-Ib also in two strains. The presence of ant(4")-Ila gene was not detected in any of tested isolates. Sixty-six products of PCR amplification were sequenced, and obtained sequences were compared with those stored in GenBank database. All sequences showed 100% similarity with sequences deposited under following accession numbers: NC_004998.1 for aac(3)-Ia, NC_004886.1 for aac(6')-Ib, M98270 for ant(4")-Ila, X04555 for ant(2")-Ia, and M28829 for aph(3")-Ib.

We analyzed resistance to eight antibiotics belonging to three groups: beta-lactams, aminoglycosides, and fluoroquinolones. All isolates showed microbiological resistance to ampicillin, amoxicillin with clavulanic acid, ceftazidime, and cefotaxime. Among aminoglycosides, MIC range of amikacin was from 4 to ≥256 μg/mL, gentamicin from 16 to ≥256 μg/mL, and tobramycin from 24 to μg/mL. According to the EUCAST interpretation tables of MIC, all strains co-producing ESBL and AME were resistant to gentamicin and tobramycin, and 53.03% of isolates was resistant to amikacin. Moreover, all strains ESBL and AME-positive were resistant to ciprofloxacin. The only antimicrobial agent, which showed 100% activity against multi-drug resistant P. mirabilis was meropenem. The results of antimicrobial susceptibility testing combined with detected genes encoding AME are presented in Table 2. Strains harboring genes encoding AME showed differentiated values of MIC50 of amikacin. The highest value of MIC50 was detected among strains carrying combination of aac(6')-Ib and aph(3")-Ib, while the lowest value showed isolates with single aph(3")-Ib gene. Strains without detected genes encoding AME were susceptible to all aminoglycosides used in this study. Strains were obtained from various clinical specimens and from numerous hospital wards. Most strains were isolated from Intensive Care Unit (32.0%), and Urology Unit (28.79%). The dominant specimen, from which strains were obtained, was urine (62.12%), followed by wound swabs (24.24%). Among patients infected by ESBL- and AME-producing P. mirabilis strains, 66.67% were males, and 33.33% were females. Patients’ mean age was 49.86 with standard deviation 8.67 years.

DISCUSSION

The present study focused on detection of plasmid-mediated AME among ESBL-positive P. mirabilis strains. In this research we observed a high rates of AME among isolates producing ESBL (88.0%). As well, Iranian survey conducted currently revealed a high frequency of AME among rods from family Enterobacteriaceae (71.0%) [21]. Data obtained from recent American assay showed that even 98.0% of K. pneumoniae resistant to carbapenems produces AME [22]. Acquisition of new resistance mechanisms by strains already resistant to particular antimicrobials creates serious concern, due to the propagation of multidrug-resistant isolates.

In our survey, the most prevalent gene encoding AME was ant(2")-Ia, present in 80.30% of AME-positive isolates. This a very interesting result, because AAC(6')-Ib is considered as the most common variant of AME among Gram-negatives, as well as Gram-positives [8,22,23]. In our previous studies conducted on P. mirabilis, and P. aeruginosa isolates, the aac(6')-Ib gene was detected in 71.43%, and 58.3% of strains, respectively [24,19]. Although, the latest research conducted in our hospital revealed the presence of ant(2")-Ia in 36.0% of strains, while the aac(6')-Ib gene was detected in smaller amount – in 28.0% of strains [18]. The resistance of clinical strains to aminoglycosides can vary with the genus of bacteria, geographic location, and many different factors. In the United States, where gentamicin is the most frequently used aminoglycoside, the gene encoding ANT(2")-Ia was detected in 81.0% of all aminoglycoside-resistant Enterobacteriaceae rods [25]. Antimicrobial susceptibility testing indicated that carbapenemers were the only drugs with full activity against all ESBL- and AME-positive strains. Other beta-lactams, like amoxicillin with clavulanic acid, cefotaxime, and ceftazidime, were inactivated by bacterial mechanisms of resistance. Also gentamicin and tobramycin showed lack of activity. Although, amikacin remained effective against 46.97% of isolates producing AME. Various level of resistance and different values of MIC50 of amikacin to AME-positive strains may be due to different levels of expression in particular isolates, and various AME substrate spectrum. In therapy of infections caused by strains producing ESBL, amikacin is usually the most active antimicrobial among aminoglycosides [26]. In the case of urinary tract sepsis caused by ESBL-positive strains susceptible to aminoglycosides, amikacin could be administered to avoid the excessive use of carbapenem [5]. Antimicrobial susceptibility testing revealed the significant share of AME in resistance to aminoglycosides among P. mirabilis strains. Results obtained in this study are consistent with the pre-
vious statements that together with the increasing prevalence of ESBL-positive strains resistant to beta-lactams, the rate of isolates resistant to aminoglycosides is also growing [27]. Infections caused by multidrug-resistant rods are associated with increased mortality than diseases caused by susceptible strains [28]. Rational management of targeted antimicrobial therapy and appropriate methods of surveillance are essential for maintaining the antibiotics activity.

**CONCLUSIONS**

In conclusion, a high frequency of simultaneous occurrence of genes encoding ESBL and AME was observed in P. mirabilis isolates. This coexistence of various mechanisms of resistance among bacterial strains significantly reduces therapeutic options. There is an urgent need for larger, multicentric survey to assess the prevalence of AME in Poland.

**ACKNOWLEDGEMENTS**

This study was supported by internal funding sources from Medical University of Bialystok (144-22822 F).

**CITE THIS AS**


**LIST OF THE TABLES**

Tab. 1. Sequences of oligonucleotide primers used in PCR assays for amplification of genes encoding AME

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Oligonucleotide sequence</th>
<th>Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>aac(3)-Ia</td>
<td>aac3-F</td>
<td>5’GGCTCTCAAAGTATGCGCATC</td>
<td>389</td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td>aac3-R</td>
<td>5’TCACCCTCAAATCTCGTGCTAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aac(A)-Ia</td>
<td>aacA4-F</td>
<td>5’GCTCTTTGAGAAGGAGGCAGG</td>
<td>300</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td>aacA4-R</td>
<td>5’TGGCTGAATCTGGCGCTG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Oligonucleotide sequence</th>
<th>Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ant(4')-Ia</td>
<td>ant4pr-F</td>
<td>5’ATGCTCTGCGAAAGGCCATAT</td>
<td>839</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td>ant4pr-R</td>
<td>5’TAAAGCGCCTATGCGTACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ant(2')-Ia</td>
<td>ant2bi-F</td>
<td>5’GACACAACGACGCTACCAT</td>
<td>500</td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td>ant2bi-R</td>
<td>5’CGCAAACCTCAACCTTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aphp(3')-Ib</td>
<td>aphp3bi-F</td>
<td>5’CTTGTGTAAGGGAATTCC</td>
<td>548</td>
<td>[20]</td>
</tr>
<tr>
<td></td>
<td>aphp3bi-R</td>
<td>5’CCAATCGCAGATAGGAAGGAAA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tab. 2. Distribution of antimicrobials MIC among P. mirabilis strains carrying combinations of genes encoding AME and without these genes

<table>
<thead>
<tr>
<th>Genes for AME</th>
<th>n</th>
<th>AMK</th>
<th>GEN</th>
<th>TOB</th>
<th>AMC</th>
<th>CTX</th>
<th>CTZ</th>
<th>MEM</th>
<th>CIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>aac(3)-Ia</td>
<td>46</td>
<td>8</td>
<td>100%</td>
<td>125</td>
<td>100%</td>
<td>88</td>
<td>100%</td>
<td>24</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
<td>100%</td>
<td>24</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aac(3)-Ia</td>
<td>5</td>
<td>32</td>
<td>100%</td>
<td>128</td>
<td>100%</td>
<td>32</td>
<td>100%</td>
<td>32</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
<td>100%</td>
<td>32</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aac(3)-Ia</td>
<td>4</td>
<td>4</td>
<td>40.0%</td>
<td>32</td>
<td>100%</td>
<td>96</td>
<td>100%</td>
<td>32</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
<td>100%</td>
<td>32</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aac(3)-Ia</td>
<td>1</td>
<td>8</td>
<td>0%</td>
<td>256</td>
<td>100%</td>
<td>32</td>
<td>100%</td>
<td>24</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>100%</td>
<td>24</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aac(3)-Ia</td>
<td>5</td>
<td>6</td>
<td>40.0%</td>
<td>256</td>
<td>100%</td>
<td>256</td>
<td>100%</td>
<td>256</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>100%</td>
<td>48</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aac(3)-Ia</td>
<td>2</td>
<td>128</td>
<td>100%</td>
<td>128</td>
<td>100%</td>
<td>128</td>
<td>100%</td>
<td>128</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>100%</td>
<td>48</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aac(3)-Ia</td>
<td>2</td>
<td>24</td>
<td>100%</td>
<td>256</td>
<td>100%</td>
<td>256</td>
<td>100%</td>
<td>256</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>100%</td>
<td>24</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aac(3)-Ia</td>
<td>9</td>
<td>2</td>
<td>0%</td>
<td>1</td>
<td>0%</td>
<td>75</td>
<td>0%</td>
<td>16</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>100%</td>
<td>24</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MEDtube Science Dec. 2014; Vol.II (4)
BIBLIOGRAPHY


